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- (71) Applicant (for all designated States except US): THER-AMED, INC. [US/US]; 9640 Medical Center Drive, Rockville, MD 20850 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): HOLADAY, John, W. [US/US]; 6502 Hillmead Road, Bethesda, MD 20815 (US). SHAH, Jamshed, H. [US/US]; 321 Haviland Mill Road, Brookeville, MD 20833 (US).
- (74) Agents: GREENE, Jamie, L. et al.; Jones & Askew, LLP, 2400 Monarch Tower, 3424 Peachtree Road, N.E., Atlanta, GA 30326 (US).
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(54) Title: COMPOSITIONS AND METHODS FOR THE INCORPORATION OF CHEMICAL SUBSTANCES INTO CELLS

(57) Abstract: The present invention relates to compositions and methods for increasing the bioavailability of chemical substances. In particular, alkylated and acetylated chemical compositions and methods for their natural incorporation into cells is provided. In one embodiment, alkylated and acetylated hemoglobin allosteric effectors are incorporated into red blood cells. These compositions and methods are useful for reducing the affinity of hemoglobin for oxygen and improving the release of oxygen from erythrocytes in tissues.

5 **COMPOSITIONS AND METHODS FOR THE INCORPORATION**
 OF CHEMICAL SUBSTANCES INTO CELLS

Technical Field

10 The present invention relates to compositions and
 methods for increasing the cellular bioavailability of chemical
 substances. More particularly, the present invention relates to
 compositions and methods for the incorporation of acetylated or
 alkylated compositions into various cell populations.

15 **Background of the Invention**

 The major function of erythrocytes, or red blood cells, is
 to transport oxygen from the lungs to the tissues of the body,
 and transport carbon dioxide from the tissues to the lungs for
 removal. The delivery of oxygen by the red blood cells is
20 accomplished through the binding of oxygen to the intracellular
 hemoglobin. Hemoglobin is a protein having a molecular
 weight of approximately 64,500 daltons. It contains four
 polypeptide chains and four heme prosthetic groups in which
 iron atoms are bound in the ferrous state. Normal globin, the
25 protein portion of the hemoglobin molecule, consists of two α
 chains and two β chains. Each of the four chains has a
 characteristic tertiary structure in which the chain is folded. The
 four polypeptide chains fit together in an approximately
 tetrahedral arrangement, to constitute the characteristic
30 quaternary structure of hemoglobin. There is one heme group
 bound to each polypeptide chain, which can reversibly bind one
 molecule of molecular oxygen. When hemoglobin combines
 with oxygen, oxyhemoglobin is formed. When oxygen is
 released, the oxyhemoglobin is reduced to deoxyhemoglobin.

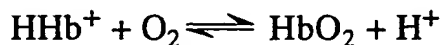
35 Delivery of oxygen to tissues depends upon a number of
 factors including, but not limited to, the volume of blood flow,
 the number of red blood cells, the concentration of hemoglobin

in the red blood cells, the oxygen affinity of the hemoglobin and, in certain species, the molar ratio of intraerythrocytic hemoglobins with high and low oxygen affinity. The oxygen affinity of hemoglobin depends on four factors as well, namely:
5 (1) the partial pressure of oxygen; (2) the pH; (3) the concentration of the allosteric effective 2,3-diphosphoglycerate (DPG) in the hemoglobin; and (4) the concentration of carbon dioxide. In the lungs, at an oxygen partial pressure of 100 mm Hg, approximately 98% of circulating hemoglobin is saturated
10 with oxygen. This represents the total oxygen transport capacity of the blood. When fully oxygenated, 100 ml of whole mammalian blood can carry about 21 ml of gaseous oxygen.

The effect of the partial pressure of oxygen and the pH on the ability of hemoglobin to bind oxygen is best illustrated
15 by examination of the oxygen saturation curve of hemoglobin. An oxygen saturation curve plots the percentage of total oxygen-binding sites of a hemoglobin molecule that are occupied by oxygen molecules when solutions of the hemoglobin molecule are in equilibrium with different partial
20 pressures of oxygen in the gas phase.

The oxygen saturation curve for hemoglobin is sigmoid. Thus, binding the first molecule of oxygen increases the affinity of the remaining hemoglobin for binding additional oxygen molecules. As the partial pressure of oxygen is increased, a
25 plateau is approached at which each of the hemoglobin molecules is saturated and contains the upper limit of four molecules of oxygen.

The reversible binding of oxygen by hemoglobin is accompanied by the release of protons, according to the
30 equation:



Thus, an increase in the pH will pull the equilibrium to the right and cause hemoglobin to bind more oxygen at a given partial
35 pressure. A decrease in the pH will decrease the amount of oxygen bound.

In the lungs, the partial pressure of oxygen in the air spaces is approximately 90 to 100 mm Hg and the pH is also high relative to normal blood pH (up to 7.6). Therefore, hemoglobin will tend to become almost maximally saturated with oxygen in the lungs. At that pressure and pH, hemoglobin is approximately 98 percent saturated with oxygen. On the other hand, in the capillaries in the interior of the peripheral tissues, the partial pressure of oxygen is only about 25 to 40 mm Hg and the pH is also relatively low (about 7.2 to 7.3). Because muscle cells use oxygen at a high rate thereby lowering the local concentration of oxygen, the release of some of the bound oxygen to the tissue is favored. As the blood passes through the capillaries in the muscles, oxygen will be released from the nearly saturated hemoglobin in the red blood cells into the blood plasma and thence into the muscle cells. Hemoglobin will release about a third of its bound oxygen as it passes through the muscle capillaries, so that when it leaves the muscle, it will be only about 64 percent saturated. In general, the hemoglobin in the venous blood leaving the tissue cycles between about 65 and 97 percent saturation with oxygen in its repeated circuits between the lungs and the peripheral tissues. Thus, oxygen partial pressure and pH function together to effect the release of oxygen by hemoglobin.

A third important factor in regulating the degree of oxygenation of hemoglobin is the allosteric effector 2,3-diphosphoglycerate (DPG). DPG is the normal physiological effector of hemoglobin in mammalian erythrocytes. DPG regulates the oxygen-binding affinity of hemoglobin in the red blood cells in relationship to the oxygen partial pressure in the lungs. In general, the higher the concentration of DPG in the cell, the lower the affinity of hemoglobin for oxygen.

When the delivery of oxygen to the tissues is chronically reduced, the concentration of DPG in the erythrocytes is increased in normal individuals. For example, at high altitudes the partial pressure of oxygen is significantly less. Correspondingly, the partial pressure of oxygen in the tissues is

less. Within a few hours after a normal human subject moves to a higher altitude, the DPG level in the red blood cells increases, causing more DPG to be bound and the oxygen affinity of the hemoglobin to decrease. This adjustment allows the hemoglobin to release its bound oxygen more readily to the tissues to compensate for the decreased oxygenation of hemoglobin in the lungs.

While DPG is the normal physiologic effector of hemoglobin in mammalian red blood cells, phosphorylated inositols are found to play a similar role in the erythrocytes of some birds and reptiles. Although inositol hexaphosphate (IHP) is unable to pass through the mammalian erythrocyte membrane in its natural state, it is capable of combining with hemoglobin of mammalian red blood cells at the binding site of DPG to modify the allosteric conformation of hemoglobin, the effect of which is to reduce the affinity of hemoglobin for oxygen. For example, DPG can be replaced by IHP, which is even more potent than DPG in reducing the oxygen affinity of hemoglobin. IHP has a 1000-fold higher affinity to hemoglobin than DPG (R.E. Benesch *et al.* 1977) and increases the P50 of hemoglobin up to values of 96.4 mm Hg at pH 7.4, and 37° C.

The low affinity conformation of the hemoglobin tetramer may be further stabilized by the addition of clofibrilic acid. Clofibrilic acid is an additional allosteric effector of hemoglobin, which when administered in conjunction with IHP, results in a cooperative reduction of the oxygen affinity for ferrous hemoglobin. (Ascenzi, P. *et al.* 1993).

The oxygen release capacity of mammalian red blood cells can be enhanced by introducing certain allosteric effectors of hemoglobin into erythrocytes, thereby decreasing the affinity of hemoglobin for oxygen and improving the oxygen economy of the blood. Improvement of the oxygen economy of the blood is particularly important for the treatment of numerous clinical conditions that involve decreased oxygenation of tissues. Examples of these clinical conditions include hypoxia, cardiovascular disease, blood loss and anemia.

5 The acute symptoms and pathology of many cardiovascular diseases, including congestive heart failure, myocardial infarction, stroke, intermittent claudication, and sickle cell anemia, result from an insufficient supply of oxygen in fluids that bathe the tissues. Likewise, the acute loss of blood following hemorrhage, traumatic injury, or surgery results in decreased oxygen supply to vital organs. Without oxygen, tissues at sites distal to the heart, and even the heart itself, cannot produce enough energy to sustain their normal functions. The result of oxygen deprivation is tissue death and organ failure.

10 One approach to alleviate the life-threatening consequences of cardiovascular disease is to increase oxygenation of tissues during acute stress. The same approach is also appropriate for persons suffering from blood loss or chronic hypoxic disorders, such as congestive heart failure. Although a physician can usually temporarily correct this condition by transfusing the patient with units of packed red blood cells; heterologous transfusions may be disfavored due to the possible scarcity of heterologous blood and the risk of infection. A safer route of treatment would be the removal of the patient's own blood, followed by the incorporation of the allosteric effector into the erythrocytes and replacement of the erythrocytes.

25 Because of the potential medical benefits to be achieved from the use of these modified erythrocytes, various techniques have been developed in the prior art to enable the encapsulation of allosteric effectors of hemoglobin in erythrocytes. Accordingly, numerous devices have been designed to assist or simplify the encapsulation procedure. The encapsulation methods known in the art include incorporation into liposomes, controlled lysis and resealing, osmotic pulse (swelling) and reconstitution of cells, and electroporation.

30 The liposome technique involves the incorporation of an allosteric effector into the liposome followed by fusion of the liposome with the red blood cell (Gersonde *et al.* 1980; Gersonde *et al.* 1982; Weiner 1983; U.S. Patent No. 4,192,869,

U.S. Patent No. 4,321,259; U.S. Patent No. 4,473,563). However, the drawbacks associated with the liposomal technique include poor reproducibility of the allosteric effector concentrations incorporated into the red blood cells and significant hemolysis of the red blood cells following treatment. Commercialization is not practical because the procedure is tedious and complicated.

Additionally, the methods of controlled lysis and resealing of red blood cells and the osmotic pulse technique have several shortcomings including low yield of encapsulation, incomplete resealing, loss of cell content and a corresponding decrease in the life span of the cells. The techniques are tedious, complicated and unsuited to automation. For these reasons, the controlled lysis and resealing and osmotic pulse techniques have had little commercial success.

Another method for encapsulating various biologically active substances in erythrocytes is electroporation. The process of electroporation involves the formation of pores in the cell membranes, or in any vesicles, by the application of electric field pulses across a liquid cell suspension containing the cells or vesicles. Electroporation has been used for encapsulation of foreign molecules including IHP in different cell types (Mouneimne, *et al.* 1990). However, as with the other prior art methods described above, electroporation un-naturally disrupts the integrity of the cell and can adversely affect cell function. Additionally, similar to the other prior art methods described above, electroporation only allows for the *in vitro* incorporation of biologically active substances into cells.

What is needed in the art are compositions and methods for the incorporation of chemical compositions into various cell types that will not severely disrupt the integrity of the cell or adversely affect cell function. Additionally, compositions and methods for the *in vivo* incorporation of chemical compositions into erythrocytes are needed to decrease the risks associated with the removal and replacement of an individual's blood.

Summary of the Invention

5 The present invention relates to compositions and methods for the incorporation of chemical compositions into various cell types. Particularly, the present invention includes methods for modifying polar chemical compositions, which in an un-modified state are not able to penetrate the lipid bilayer of a cell. The resulting chemical compositions are naturally incorporated into the cell. Thus, the compositions and methods of the present invention are useful for increasing the
10 bioavailability of polar chemical substances.

In one embodiment, the present invention provides compositions and methods for the incorporation of allosteric effectors of hemoglobin into erythrocytes. A preferred
15 allosteric effector is an inositol-hexasodiumphosphate-hexa-alkylester, however, other similarly modified inositols may also be used. An alternate allosteric effector of hemoglobin included in the present invention is clofibrate as modified by the methods of the present invention.

The compositions and methods of the present invention
20 overcome the problems in the prior art including loss of cell content and disruption of cell function upon incorporation of chemical substances into cells. Unlike the prior art methods, the present invention provides a means to incorporate chemical substances into various cells, including red blood cells, via
25 natural up-take mechanisms. In one embodiment of the present invention, modified hemoglobin allosteric effector compositions are incorporated into erythrocytes *in vitro*, and the erythrocytes are subsequently administered to an individual. In an alternate embodiment, modified hemoglobin allosteric effector
30 compositions are administered directly to the individual, whereupon the compositions are incorporated into erythrocytes *in vivo*.

In another embodiment of the present invention, chemicals used in the preservation of erythrocytes are
35 incorporated into erythrocytes *in vitro* and the erythrocytes are subsequently administered to an individual. In a preferred embodiment, trehalose is modified by the methods of the

present invention and incorporated into erythrocytes. Alkylated or acetylated trehalose may be administered to an erythrocyte alone or in combination with additional substances. Additional substances include, but are not limited to, other chemicals used in the preservation of erythrocytes such as DMSO, and the modified hemoglobin allosteric effector compositions as described herein.

The compositions and methods of the present invention are useful for the treatment of several conditions and diseases, including, but not limited to, those associated with decreased oxygenation of tissues. The incorporation of allosteric effector compositions into red blood cells lowers the oxygen affinity of the erythrocytes and increases the capacity of erythrocytes to dissociate the bound oxygen and thereby improves the oxygen supply to the tissues. Enhancement of the oxygen-release capacity of erythrocytes brings about significant physiological effects such as a reduction in cardiac output, an increase in the arteriovenous differences, and improved tissue oxygenation.

The modified erythrocytes prepared in accordance with the present invention, having improved oxygen release capacities, may find their use in situations such as those illustrated below:

1. Under conditions of low oxygen-partial pressure, such as at high altitudes;
2. During autologous blood procurement techniques such as hemodilution;
3. When the oxygen exchange surface of the lung is reduced, such as occurs in emphysema;
4. When there is an increased resistance to oxygen diffusion in the lung, such as occurs in pneumonia or asthma;
5. When there is a decrease in the oxygen-transport capacity of erythrocytes, such as occurs with erythropenia or anemia, or when an arteriovenous shunt is used;
6. To treat blood circulation disturbances, such as arteriosclerosis, thromboembolic processes, organ infarct, congestive heart failure, cardiac insufficiency or ischemia;

7. To treat conditions of high, oxygen affinity of hemoglobin, such as hemoglobin mutations, chemical modifications of N-terminal amino acids in the hemoglobin-chains, or enzyme defects in erythrocytes;

5 8. To accelerate detoxification processes by improving oxygen supply;

9. To decrease the oxygen affinity of conserved blood; or

10 10. To improve the efficacy of various cancer treatments.

Thus, it is an object of the present invention to provide compositions and methods for increasing the bioavailability of chemical substances.

15 It is a another object of the present invention to provide compositions and methods for the incorporation of polar chemical substances into cells via alkylation or acetylation of the chemical substances.

20 It is a further object of the present invention to provide compositions and methods for the incorporation of allosteric effectors of hemoglobin into erythrocytes.

It is another object of the present invention to provide compositions and methods for the incorporation of preservatives of erythrocytes into erythrocytes.

25 It is another object of the present invention to provide a method for the *in vivo* incorporation of modified inositol phosphates into erythrocytes.

30 It is another object of the present invention to provide an improved method for the incorporation of polar chemical substances that reduces, if not abrogates, loss of cell content and loss of cell function.

It is a further object of the present invention to provide an improved method for the incorporation of allosteric effectors of hemoglobin that reduces, if not abrogates, loss of erythrocyte cell content and loss of erythrocyte function.

35 It is a further object of the present invention to provide a composition suitable for use in the treatment of conditions

and/or disease states resulting from a lack of or decrease in oxygenation.

5 These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

Detailed Description of the Invention

10 The present invention relates to compositions and methods for the incorporation of chemical substances into various cell populations. In particular, polar chemicals that cannot naturally traverse the lipid bilayer of a cell membrane are modified in such a manner that the chemicals may then be incorporated into the cell without unnaturally disrupting the
15 cellular membrane. It is to be understood that the present invention is not limited to a particular polar chemical substance, and any polar chemical substance may be modified by the methods of the present invention. It is also to be understood that the terms "bioavailable", "modified" and "modification"
20 when referring to a chemical substance refer to the addition of chemical side chains such that the resulting chemical composition is sufficiently less polar and has an improved ability to traverse the lipid bilayer of a cell as compared to the initial chemical substance. Examples of the methods of modification
25 of polar chemical substances described herein include alkylation and acetylation of the polar chemical substances. The alkyl or acetyl groups may contain from 1-18 carbon atoms and may contain straight or branched alkyls, cycloalkyls, alkenyls, or cycloalkenyls.

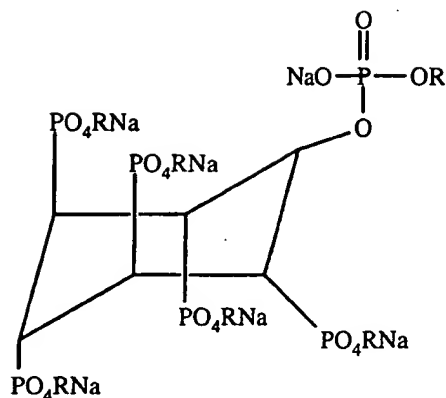
30 Although not wanting to be bound by this hypothesis, it is believed that alkylation or acetylation of a polar chemical decreases its polar nature and causes it to become more lipophilic. This change in polarity facilitates incorporation of the chemical into the lipid bilayer of the cell and results in the
35 natural incorporation of the chemical into the cell. In order for many of the modified chemicals to be biologically active, the alkyl and acetyl groups must be removed by the appropriate

enzymes once the chemical is inside the cell. Therefore, in a preferred embodiment, the cells of the present invention contain enzymes capable of removing alkyl and acetyl groups.

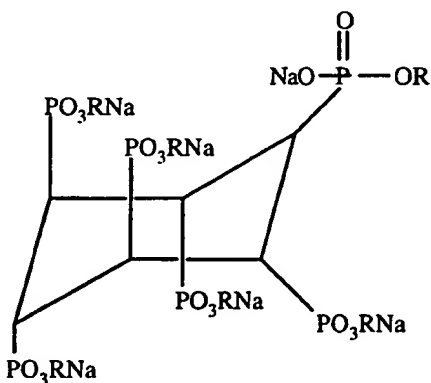
5 In one embodiment of the present invention, the compositions and methods described herein are used to incorporate allosteric effectors of hemoglobin into erythrocytes. The compositions and methods provided herein are useful for the treatment of conditions and diseases associated with decreased oxygenation of tissues.

10 One advantage of hemoglobin allosteric effector-treated red blood cells provided in the present invention is that they do not lose the Bohr effect when stored. Normal red blood cells that have been stored by conventional means do not regain their maximum oxygen carrying capacity for approximately 24
15 hours. This is because the DGP in normal red blood cells diffuses away from the hemoglobin molecule during storage and must be replaced by the body after transfusion. In contrast, red blood cells treated according to the present invention retain their maximum oxygen carrying capacity during storage and
20 therefore can deliver maximum oxygen to the tissues immediately after transfusion into a human or animal.

According to the present invention, one preferred modified allosteric effector of hemoglobin is an inositol-hexasodiumphosphate-hexa-alkylester with the following general
25 chemical structure.



- 5 Another preferred modified allosteric effector of hemoglobin is an inositol-hexasodiumphosphonate-hexa-alkylester with the following general chemical structure:



10

- 15 As discussed above, the modified IHP compounds shown above incorporate into the red blood cell naturally, without negatively affecting cellular functions. Upon internalization of the modified IHP compound, the enzymes in the red blood cell remove the alkyl or acetyl side groups and produce an active IHP compound. The active IHP compound is then able to bind to the intracellular hemoglobin and decrease the hemoglobin's affinity for oxygen. Since the above described incorporation is

a natural process and does not disrupt the integrity of the erythrocyte, loss of cell content is minimalized, if not abrogated.

It is to be understood that the present invention is not limited to the IHP compounds described above. The present invention also encompasses the modification of any polar chemical that cannot naturally traverse the lipid bilayer of a cell membrane. In particular, the present invention encompasses other alkylated hemoglobin allosteric effector compositions. The term "alkylated hemoglobin allosteric effector composition" as used herein includes, but is not limited to, other sugar phosphates that may be modified by the methods of the present invention and incorporated into erythrocytes as allosteric effectors of hemoglobin. Examples of alkylated hemoglobin allosteric effector compositions are inositol pentasodiumphosphate-penta-alkyl ester, inositol tetrasodiumphosphate-tetra-alkyl ester, inositol trisodiumphosphate-trialkyl ester, and inositol disodiumphosphate-dialkyl ester, diphosphatidyl inositol diphosphate, polyphosphates such as nucleotide triphosphates, nucleotide diphosphates, nucleotide monophosphates, and alcohol phosphate esters, organic anions such as polycarboxylic acids that can be used in case of certain mutations of hemoglobin, e.g. "Zurich" hemoglobin, and inorganic anions such as hexacyano ferrate, phosphate or chloride.

Another example of a hemoglobin allosteric effector composition included in the present invention is clofibrate as modified by the methods described herein. In one embodiment of the present invention, clofibrate is alkylated or acetylated and incorporated into erythrocytes *in vitro* or *in vivo* by the methods of the present invention. In a preferred embodiment, the alkyl or acetyl groups attached to the clofibrate are removed by enzymes inside the erythrocyte following incorporation of clofibrate. It is to be understood that modified clofibrate may be administered to erythrocytes in conjunction with additional biologically active substances, including but not limited to, other modified allosteric effectors of hemoglobin such as the IHP compounds described herein.

In another embodiment of the present invention, chemicals used in the preservation of erythrocytes are modified and incorporated into erythrocytes *in vitro* and the erythrocytes are subsequently administered to an individual. In a preferred
5 embodiment, trehalose is modified by the methods of the present invention and incorporated into erythrocytes. In one embodiment, trehalose is alkylated. In another embodiment, trehalose is acetylated. Bioavailable trehalose may be administered to the erythrocyte alone or in combination with
10 additional substances. Additional substances include, but are not limited to, other chemicals used in the preservation of erythrocytes such as DMSO, and the modified hemoglobin allosteric effector compositions as described herein.

The bioavailable preservative compositions described
15 above are particularly useful during autologous blood procurement techniques such as hemodilution. Blood removed from the patient is mixed with modified chemicals such as trehalose and the modified chemical is allowed to incorporate into the blood cells. The blood may then be stored for extended
20 periods of time before re-introducing the blood into the patient. In one embodiment of the present invention, both alkylated or acetylated trehalose and an alkylated or acetylated hemoglobin allosteric effector composition are incorporated into the blood cells. Incorporation of both a bioavailable preservative
25 composition and a bioavailable hemoglobin allosteric effector composition allows for the preservation of red blood cells for an extended period of time combined with an increased oxygenation of tissues upon their re-introduction into the patient.

30 The bioavailable compositions described above may also be conjugated to, or labeled by, other molecules. When labeled with a detectable biomolecule or chemical, the compositions described above are useful for purposes such as *in vivo* and *in vitro* diagnostics and laboratory research using methods and
35 assays well known in the art. For example, the modified compositions are conjugated to a radiolabel such as, but not restricted to, ^{32}P , ^3H , ^{14}C , ^{35}S , ^{125}I , or ^{131}I . Detection of a

label can be by methods such as scintillation counting, gamma ray spectrometry or autoradiography. Fluorogens may also be used as labels. Examples of fluorogens include fluorescein and derivatives, phycoerythrin, allo-phycoerythrin, phycoerythrin, rhodamine, and Texas Red. The fluorogens are generally detected by a fluorescence detector. The modified compositions of the present invention may also be labeled with antibodies that are conjugated to a detectable biomolecule or chemical, including, but not limited to, a chromogen, fluorogen, bioluminescent label, radiolabel, or colloidal gold.

The compositions modified by the methods of the present invention are useful for the treatment of conditions and diseases associated with decreased oxygenation of tissues. Conditions or diseases that may be treated by the administration of the modified compositions, and particularly the modified hemoglobin allosteric effectors of the present invention, to red blood cells include, but are not limited to, heart attack, "bleeding" anemia, surgical complications, stroke, diabetes, sickle cell disease, burns, intermittent claudication, emphysema, hypothermia, peripheral vascular disease, congestive heart failure, angina, transient ischemic disease, disseminated intravascular coagulation, adult respiratory distress syndrome (ARDS) and cystic fibrosis.

When appropriate, the bioavailable compositions described above may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and pharmaceutical carrier(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The bioavailable compositions may be administered in aqueous suspension, oil emulsion, water in oil emulsion, water-in-oil-in-water emulsion, liposomes, microparticles, site-specific emulsions, long-residence emulsions, sticky-emulsions, microemulsions, nanoemulsions, microspheres, nanospheres,

nano-particles, minipumps, and with various natural or synthetic polymers that allow for sustained release of the composition. Further, the acetylated compositions can be used with any one, all, or any combination of excipients regardless of the carrier used to present the composition to the responding cells. These include, but are not limited to, anti-oxidants, buffers, and bacteriostats, and may include suspending agents and thickening agents.

For administration in an aqueous carrier, the bioavailable composition is suspended in a pharmaceutically acceptable carrier such as, but not limited to, water, saline or phosphate buffered saline (PBS) and is emulsified by sonication. Optionally, the emulsified mixture is homogenized by microfluidization. For administration in a non-aqueous carrier, the bioavailable composition is emulsified with a mineral oil or with a neutral oil such as, but not limited to, a diglyceride, a triglyceride, a phospholipid, a lipid, an oil and mixtures thereof, wherein the oil contains an appropriate mix of polyunsaturated and saturated fatty acids. Examples include, but are not limited to, squalane, squalene, the synthetic mineral oil n-hexadecane and to soybean oil, canola oil, palm oil, olive oil and myglyol, wherein the number of fatty acid carbons is between 12 and 22 and wherein the fatty acids can be saturated or unsaturated. Optionally, charged lipid or phospholipid can be suspended in the neutral oil. It will be understood by those skilled in the art that the method of preparing the emulsion is not critical. Numerous variations of the composition of the oil and aqueous phases, their proportions and means of emulsification will be apparent to those skilled in the art and can be used with the bioavailable compositions in practicing the methods of the present invention described below.

In one embodiment of the present invention, the bioavailable compositions described above are administered to red blood cells *in vitro*, and following uptake of the hemoglobin allosteric effector by the red blood cells, the red blood cells are administered to an individual. In a preferred embodiment the red blood cells are autologous cells, however the red blood cells

may also be heterologous to the individual. In a further preferred embodiment, the erythrocytes are washed and resuspended in a solution of the biologically active substance to be introduced into the cell and then incubated. Following incubation, the cells are washed and separated. A contamination check is optionally conducted to confirm that all un-encapsulated biologically active substance has been removed. Then, the cells are prepared for storage or re-introduction into a patient.

Treating a human or animal for any one or more of the disease states described in more detail below may be accomplished by transfusing into the human or animal between approximately 0.5 and 6 units (1 unit = 500 ml) of hemoglobin allosteric effector-treated blood that has been prepared according to the present invention. In certain cases, there may be a substantially complete replacement of all the normal blood in a patient with hemoglobin allosteric effector-treated blood. The volume of treated red blood cells that is administered to the human or animal will depend upon the indication being treated. In addition, the volume of treated red blood cells will also depend upon concentration of hemoglobin allosteric effector-treated red blood cells in the red blood cell suspension. It is to be understood that the quantity of red blood cells treated according to present invention that is administered to the patient is not critical and can vary widely and still be effective.

Hemoglobin allosteric effector-treated packed erythrocytes are similar to normal red blood cells in except that the treated packed red blood cells can deliver 2 to 3 times as much oxygen to tissue per unit. A physician would therefore chose to administer a single unit of hemoglobin allosteric effector-treated packed red blood cells rather than two units of the normal red blood cells. Hemoglobin allosteric effector-treated packed red blood cells could be prepared in blood processing centers analogously to the present blood processing methods, except for the inclusion of a processing step where the hemoglobin allosteric effector is introduced into the red blood cell.

In an alternate embodiment of the present invention, when appropriate and non-toxic, the bioavailable compositions described above may be administered directly to an individual, whereupon the compositions are internalized by the erythrocytes *in vivo*. When directly administering the bioavailable composition, the formulations can be administered by standard routes. In general, the compositions may be administered by the transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (e.g., intravenous, intraspinal, subcutaneous or intramuscular) route. Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules, vials or containers, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

For treating humans or animals with a direct administration of the bioavailable compositions of the present invention, between approximately 0.5 mg/kilogram to 500 mg/kilogram of a bioavailable composition can be administered. A more preferable range is 1 mg/kilogram to 100 mg/kilogram with the most preferable range being from 2 mg/kilogram to 50 mg/kilogram. Depending upon the half-life of the bioavailable composition in the particular animal or human, it can be administered between several times per day to once a week. It is to be understood that the present invention has application for both human and veterinary use. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time.

The methods of the present invention include methods of treating diseases and conditions associated with decreased oxygenation of tissues comprising the administration of hemoglobin allosteric effector compositions to an individual having the disease or condition. In one embodiment, the hemoglobin allosteric effector compositions of the present invention are administered to a patient undergoing a heart attack, thereby increasing the oxygen delivery to the ischemic heart tissue and, at the same time, reducing the cardiac output.

The hemoglobin allosteric effector compositions of the present invention may also be administered to an individual having any ischemic condition including, but not limited to, "bleeding" anemia, surgical complications, diabetes, burns, emphysema, hypothermia, angina, transient ischemic disease, disseminated intravascular coagulation, adult respiratory distress syndrome (ARDS), cystic fibrosis, cardiovascular operations, chronic anemia, anemia following major surgery, coronary infarction and associated problems, chronic pulmonary disease, cardiovascular disease, autologous transfusions, as an enhancement to packed red blood cells transfusion (hemorrhage, traumatic injury, or surgery), congestive heart failure, myocardial infarction (heart attack), stroke, peripheral vascular disease, intermittent claudication, circulatory shock, hemorrhagic shock, chronic hypoxmia, respiratory alkalemia, metabolic alkalosis, sickle cell anemia, reduced lung capacity caused by pneumonia, surgery, trauma, chest puncture, gangrene, anaerobic infections, blood vessel diseases such as diabetes, substitute or complement to treatment with hyperbaric pressure chambers, intra-operative red cell salvage, cardiac inadequacy, anoxia - secondary to chronic indication, organ transplant, carbon monoxide, nitric oxide, and cyanide poisoning.

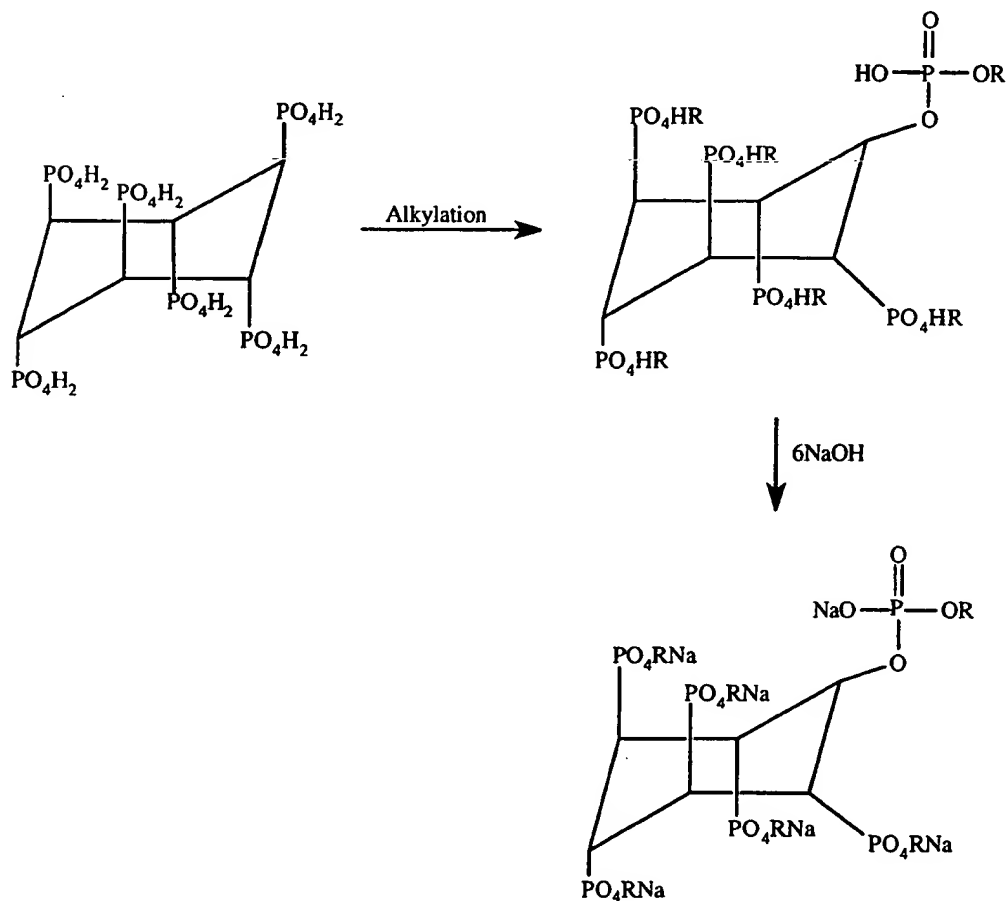
As described above, the bioavailable hemoglobin allosteric effector compositions may be administered to red blood cells *in vitro* prior to their introduction into the individual or the compositions may be introduced into the individual directly. The dosage of a hemoglobin allosteric effector of the

present invention will depend upon the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose, as herein above recited, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients, particularly mentioned above, the formulations of the present invention may include other agents conventional in the art having regard to the type of formulation in question.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

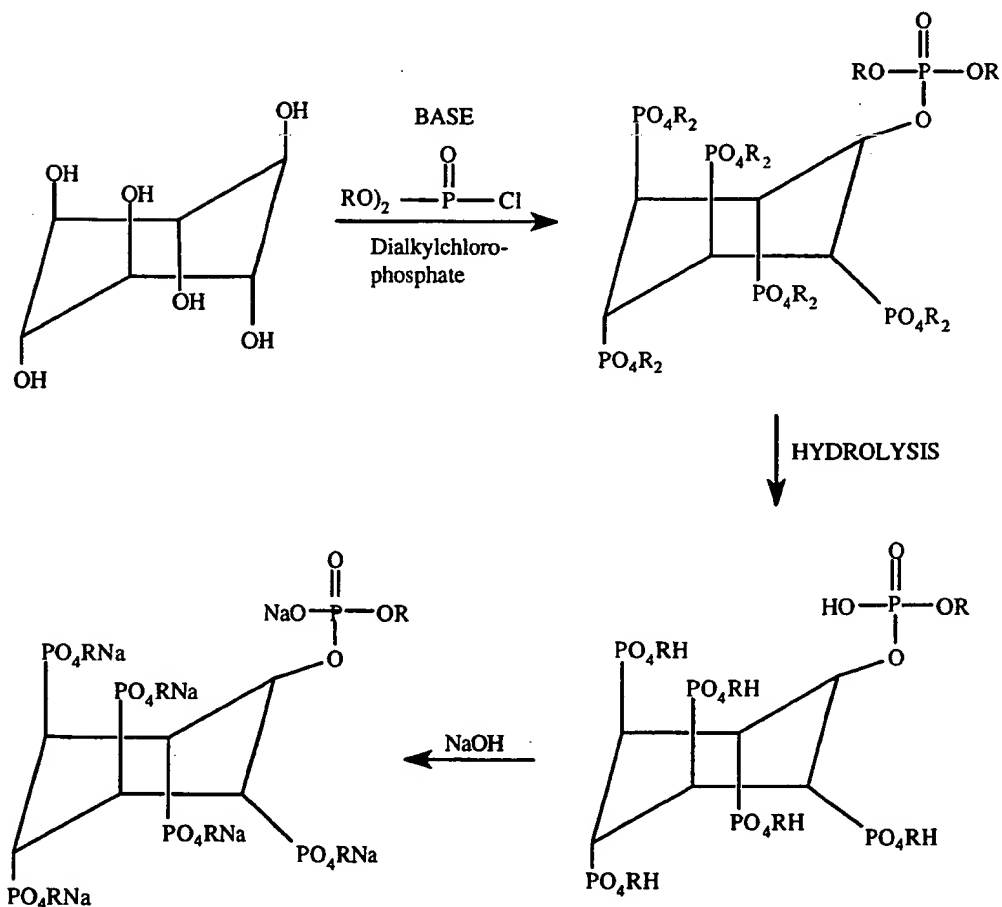
Example 1*Preparation of Inositol-hexasodiumphosphate-hexa-alkylester*

- 5 Inositol-hexasodiumphosphate-hexa-alkylester is prepared according to the following chemical reaction wherein R is CH_3 , CH_3CH_2 , Ph, PhCH_2 , $(\text{CH}_2)_2\text{CH}_3$, or $\text{C}(\text{CH}_3)_3$.



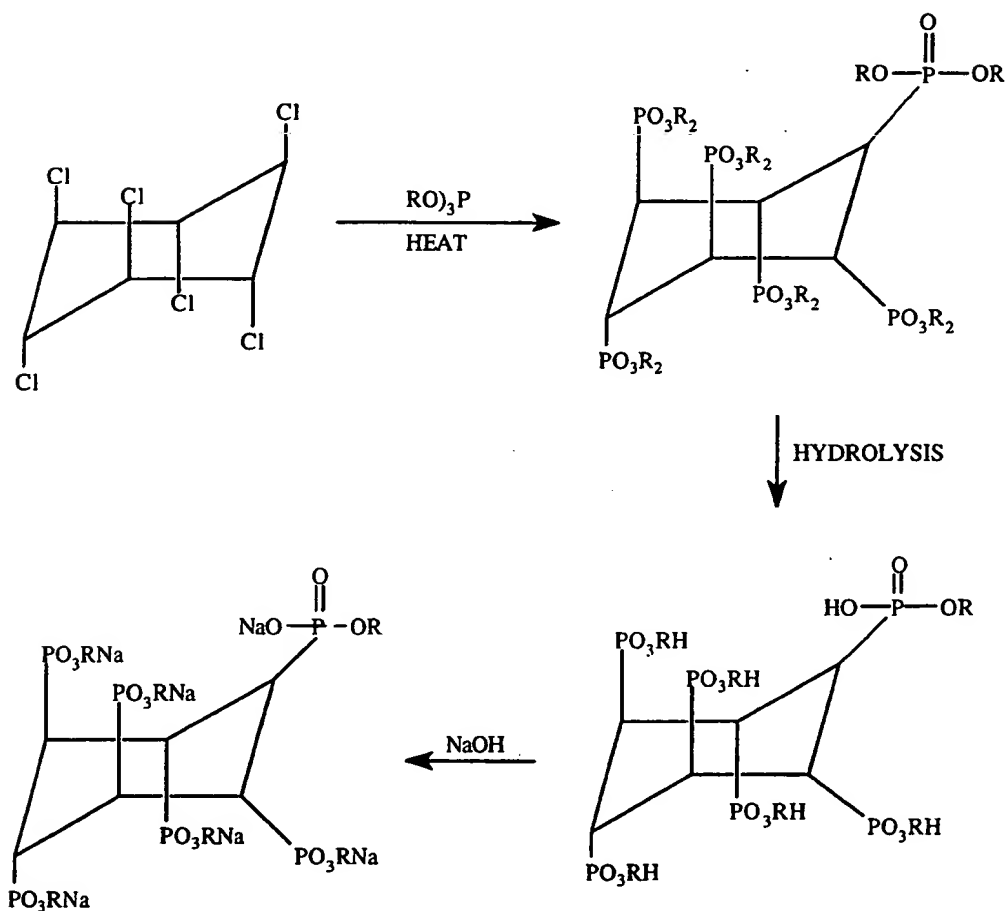
Example 2*Alternative Method for the Preparation of Inositol-hexasodiumphosphate-hexa-alkylester*

- 5 Inositol-hexasodiumphosphate-hexa-alkylester is prepared according to the following chemical reaction wherein R is CH₃, CH₃CH₂, Ph, PhCH₂, (CH₂)₂CH₃, or C(CH₃)₃.



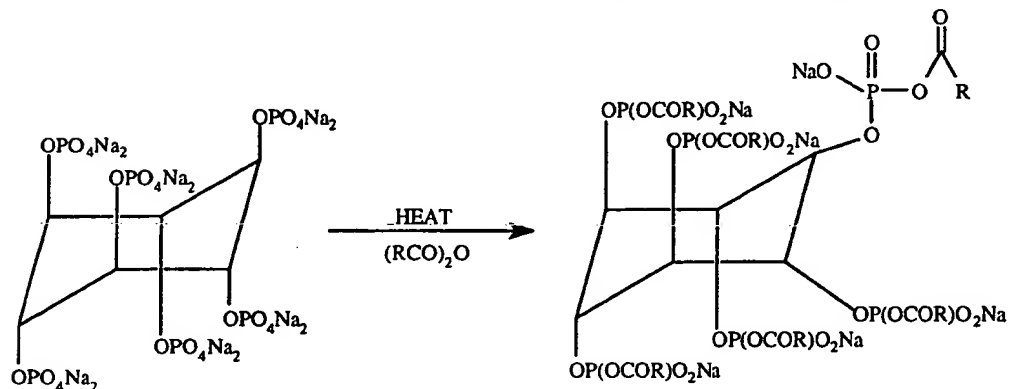
Example 3*Method for the Preparation of Inositol-hexasodiumphosphonate-hexa-alkylester*

- 5 Inositol-hexasodiumphosphonate-hexa-alkylester is prepared according to the following chemical reaction wherein R is CH₃, CH₃CH₂, Ph, PhCH₂, (CH₂)₂CH₃, or C(CH₃)₃.



Example 4*Method for the Preparation of Inositol-hexaphosphoric acid-hexalkyltyle sodium salt*

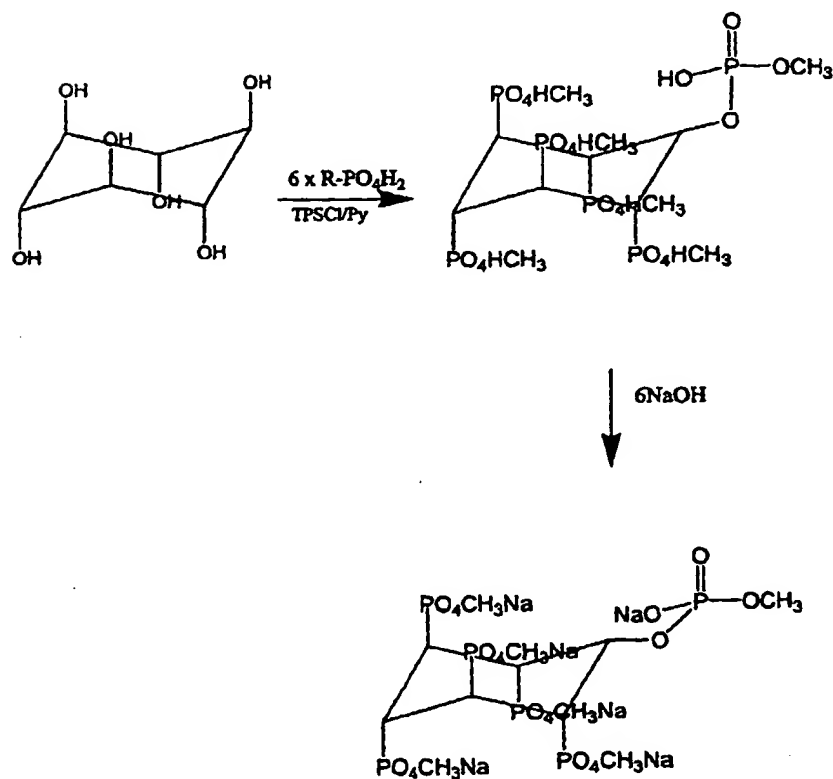
- 5 Inositol-hexaphosphoric acid-hexalkyltyle sodium salt is prepared according to the following chemical reaction wherein R is CH₃, CH₃CH₂, Ph, PhCH₂, (CH₂)₂CH₃, or C(CH₃)₃.



Example 5

Method for the Preparation of Inositol-hexasodiumphosphate hexamethylester

- 5 Inositol-hexasodiumphosphate hexamethylester is prepared according to the following chemical reaction wherein R is CH₃, CH₃CH₂, Ph, PhCH₂, (CH₂)₂CH₃, C₆H₄CH₃, C₆H₄Ph, CH(CH₃)₂, C_nH_{2n}CH₃, C_nH_{2n}Ph, CH(CH₃)CH₂CH₃, or C(CH₃)₃.



5 While this invention has been described in specific detail with reference to the disclosed embodiments, it will be understood that many variations and modifications may be effected within the spirit and scope of the invention as described in the appended claims.

Claims

What is claimed is:

- 5 1. A composition comprising a modified extracellular chemical having the ability to penetrate a cellular membrane of biological cells, wherein the chemical, prior to modification, lacks the ability to penetrate the cell membrane.
- 10 2. The composition of Claim 1, wherein the chemical is a polar chemical substance.
3. The composition of Claim 1, wherein the chemical is an allosteric effector of hemoglobin.
- 15 4. The composition of Claim 1, wherein the biological cells are erythrocytes, lymphocytes or platelets.
5. The composition of Claim 1, wherein the chemical penetrates the cellular membrane by electroporation.
- 20 6. The composition of Claim 1, wherein the chemical is modified by alkylation or acetylation.
7. The composition of Claim 1, wherein the modification of the chemical is performed *in vitro* or *in vivo*.
- 25 8. The composition of Claim 1, wherein the modified chemical is selected from the group consisting of inositol-hexaphosphate-hexalkylesters, other modified inositols, alkylated or acetylated trehalose, DMSO, and blood preservative chemicals.
- 30

9. The composition of Claim 1, wherein the modified chemical, when administered to a human or animal, produces a physiological effect.
- 5
10. The composition of Claim 1, wherein the physiological effect is selected from the group consisting of reduced cardiac output and improved oxygen release capacities.
- 10
11. A method for increasing the bioavailability of an extracellular chemical comprising:
- 15
- chemically modifying the extracellular chemical to have the ability to penetrate the cellular membrane of a biological cell, wherein the chemical is normally unable to penetrate the cellular membranes of biological cells; and
- reacting the modified chemical with biological cells to cause intracellular incorporation of the chemical by penetration of the cellular membrane by the chemical.
- 20
12. The method of Claim 11, wherein the chemical is a polar chemical substance.
13. The method of Claim 11, wherein the chemical compound is an allosteric effector of hemoglobin.
- 25
14. The method of Claim 11, wherein the biological cells are erythrocytes, lymphocytes or platelets.
15. The method of Claim 11, wherein the chemical is reacted with the cells by electroporation.
- 30

16. The method of Claim 11, wherein the chemical is modified by alkylation or acetylation.

5 17. The method of Claim 11, wherein the modification of said chemical compounds is accomplished *in vitro* or *in vivo*.

10 18. The method of Claim 11, wherein the modified chemical is selected from the group consisting of inositol-hexaphosphate-hexalkylesters, other modified inositols, alkylated or acetylated trehalose, DMSO, and blood preservative chemicals.

15 19. The composition of Claim 11, wherein the modified chemical, when administered to a human or animal, produces a physiological effect.

20 20. The composition of Claim 11, wherein the physiological effect is selected from the group consisting of reduced cardiac output and improved oxygen release capacities.